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Full Length Research Paper

Evaluation of the Antimicrobial Activity and Efficacy of Natural Preservatives Against Spoilage Microorganisms in Fermented Dairy Products

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ABSTRACT
This study investigated the preservative efficacy of six natural compounds, clove essential oil, nisin, infused olive oil, garlic extract, ginger extract, and extra virgin olive oil, across five culturally significant fermented dairy products: yogurt (FDP-01), kefir (FDP-02), paneer (FDP-03), shrikhand (FDP-04), and misti doi (FDP-05). The objective was to evaluate their antimicrobial, physicochemical, and sensory impact over a 21-day refrigerated storage period, with emphasis on microbial suppression, shelf-life extension, and consumer acceptability.
Microbial screening was performed against dominant spoilage organisms, including Bacillus subtilis, Pseudomonas fluorescens, Escherichia coli, Candida albicans, and Penicillium spp. Zone of inhibition assays, MIC/MBC/MFC determinations, and multivariate ranking revealed clove essential oil and nisin as the most potent agents, with MIC values ≤0.5 mg/mL and complete inhibition of fungal growth at low concentrations. Their efficacy was further validated through declining total viable counts (TVC) in treated samples, maintaining microbial loads below spoilage thresholds throughout storage.
Physicochemical parameters (pH, titratable acidity, viscosity) were monitored at 7-day intervals using standard methods. Control samples exhibited progressive acidification and viscosity loss, while clove oil and nisin treatments preserved structural integrity across all matrices. In paneer and misti doi, nisin proved particularly effective in minimizing curd separation and proteolytic degradation. Strong inverse correlations were observed between microbial proliferation and both pH (r = –0.88) and viscosity (r = –0.76), affirming the biochemical role of microbial control in spoilage prevention.
Keywords: *Natural preservatives, Fermented dairy, Clove essential oil, Nisin, Shelf-life extension, Sensory evaluation*

INTRODUCTION

Fermented dairy products have long held a central role in human nutrition, offering a unique combination of sensory appeal, nutritional enhancement, and microbial functionality. These products such as yogurt, kefir, cheese, and dahi are created through controlled microbial fermentation of milk, primarily involving lactic acid bacteria (LAB) like *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Panesar, 2011). The fermentation process not only improves digestibility and flavor but also extends shelf life by lowering pH and inhibiting spoilage organisms (Saleem et al., 2024).

In recent decades, consumer preferences have shifted toward minimally processed, additive-free foods, prompting renewed interest in natural preservation strategies. Conventional synthetic preservatives, while effective, have raised concerns regarding toxicity, allergenicity, and long-term health effects (Thakre, 2023). This has catalyzed research into plant-derived antimicrobials, essential oils, and bacteriocins as safer alternatives for food preservation, particularly in dairy matrices where microbial stability is critical.

The present study aims to evaluate the antimicrobial activity and efficacy of selected natural preservatives against spoilage microorganisms in fermented dairy products. By integrating microbiological assays, shelf-life simulations, and sensory evaluations, this research seeks to contribute to sustainable food preservation practices and enhance product safety without compromising quality.

Importance of Fermented Dairy Products

Fermented dairy products are considered “nutritional pearls” of the food industry due to their enhanced bioavailability of nutrients, probiotic potential, and therapeutic benefits (Saleem et al., 2024). The fermentation process transforms lactose into lactic acid, creating an acidic environment that inhibits pathogenic growth and supports beneficial

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microbial populations (Carocho & Ferreira, 2021). This transformation also leads to the synthesis of bioactive compounds such as peptides, vitamins (e.g., B12, K2), and short-chain fatty acids, which contribute to gut health, immune modulation, and metabolic regulation (Panesar, 2011; Naz et al., 2024).

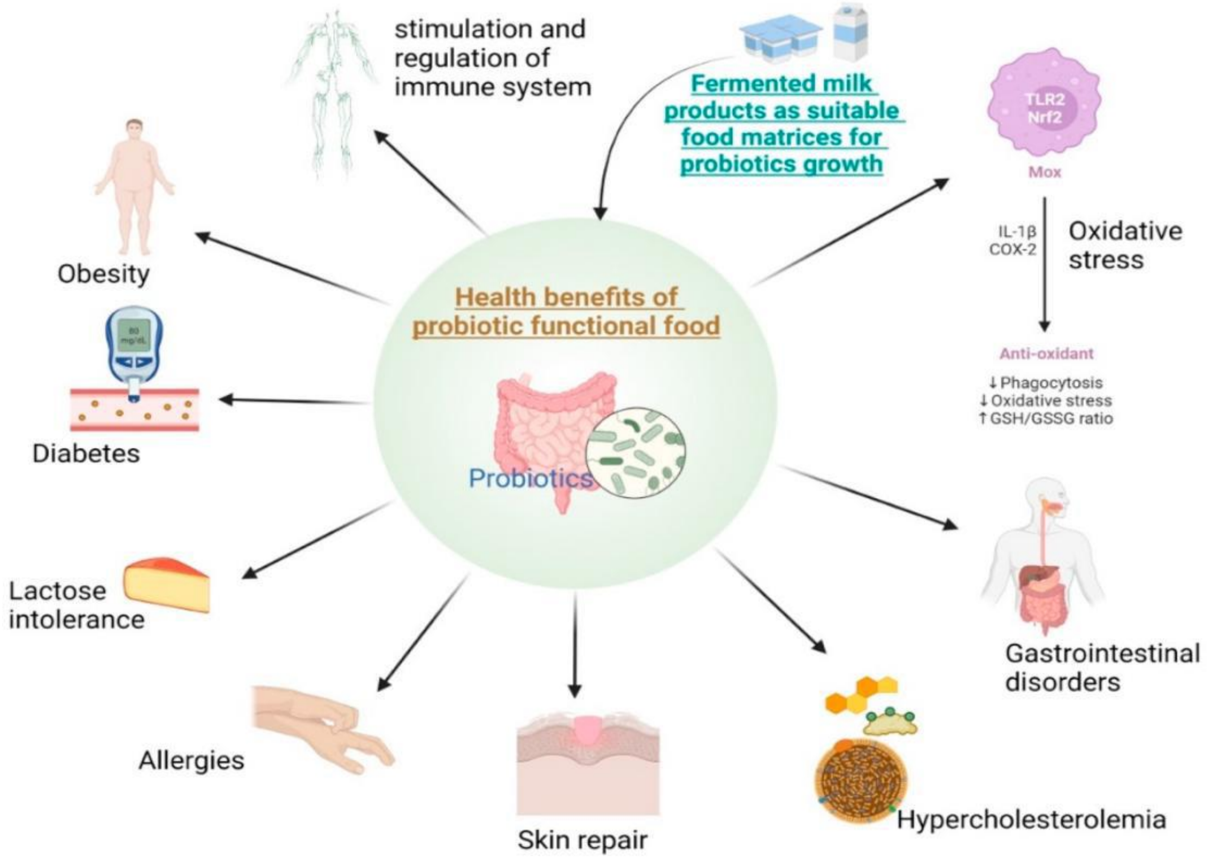

Fig 1: Fermented dairy-based product health benefits

CHAPTER II -REVIEW OF LITERATURE

Overview of Fermented Dairy Products

Fermented dairy products represent one of the oldest and most culturally significant categories of functional foods. Their origins trace back thousands of years, with early civilizations using spontaneous fermentation to preserve milk and enhance its digestibility (Panesar, 2011). Today, these products ranging from yogurt and kefir to cheese and dahi are produced through controlled microbial fermentation, primarily involving lactic acid bacteria (LAB) such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Lactococcus lactis* (Puniya et al., 2015).

The fermentation process transforms lactose into lactic acid, lowering the pH and creating an environment that inhibits spoilage organisms while promoting the growth of beneficial microbes (Dairy Processing Handbook, 2024). This biochemical shift not only extends shelf life but also contributes to the development of characteristic flavors, textures, and nutritional enhancements. For example, fermentation can increase the bioavailability of vitamins (e.g., B12, K2), generate bioactive peptides, and reduce lactose content—making these products more suitable for lactose-intolerant individuals (Panesar, 2011; Okoniewski et al., 2023).

Globally, fermented dairy products are consumed in diverse forms. Yogurt is a staple in many Western and Asian diets, typically produced by fermenting milk with *S. thermophilus* and *L. bulgaricus* at elevated temperatures (IDF, 2024). Kefir, originating from the Caucasus region, is a complex fermented milk beverage made using kefir grains a symbiotic matrix of bacteria and yeasts, that yields both lactic acid and mild alcohol, contributing to its effervescent character and probiotic potential (Schwan & Freitas, 2015).

Cheese, perhaps the most diverse fermented dairy category, involves coagulation of milk proteins followed by ripening processes that introduce secondary microflora such as *Penicillium roqueforti* and *Propionibacterium freudenreichii*, which contribute to flavor complexity and texture development (Puniya et al., 2015). In India, traditional products like dahi and lassi are prepared using indigenous starter cultures, often passed down through generations, and hold both culinary and cultural significance.

The nutritional profile of fermented dairy products is enhanced through microbial metabolism. Proteins are partially hydrolyzed into peptides and amino acids, fats undergo lipolysis, and carbohydrates are converted into organic acids and volatile compounds that contribute to flavor and health benefits (Panesar, 2011; Puniya et al., 2015). These transformations also support gut health, immune modulation, and cardiovascular function, positioning fermented dairy as a cornerstone of functional nutrition.

In summary, fermented dairy products exemplify the intersection of tradition, microbiology, and nutrition. Their continued relevance in modern diets underscores the importance of preserving microbial integrity and exploring innovative preservation strategies such as natural antimicrobials to maintain safety and quality in an increasingly health-conscious market.

Yeasts

Yeasts such as *Candida spp.*, *Kluyveromyces marxianus*, and *Debaryomyces hansenii* are prevalent in products like kefir, lassi, and flavored yogurts. They ferment residual sugars and organic acids, producing alcohol, carbon dioxide, and off-flavors. Yeast spoilage is often characterized by effervescence, fruity odors, and surface film formation (Xiao & Li, 2022).

Molds

Molds including *Penicillium spp.*, *Cladosporium spp.*, *Aspergillus spp.*, and *Mucor spp.* typically colonize the surface of dairy products, especially those with added fruits or nuts. They produce visible colonies, discoloration, and mycotoxins under favorable conditions. Mold spoilage is particularly problematic in soft cheeses and yogurt, where moisture and oxygen exposure facilitate fungal growth (Thapa Magar, 2022).

Spore-Forming Bacteria

Spore-formers such as *Bacillus spp.* and *Clostridium tyrobutyricum* survive pasteurization and can germinate during storage. *C. tyrobutyricum* is notorious for causing late gas defects in cheese, producing butyric acid and hydrogen gas that lead to bloating and off-odors. *Bacillus cereus* can cause sweet curdling and bitty cream defects due to proteolytic activity (Ledenbach & Marshall, 2009).

CHAPTER III-METHODOLOGY

Sampling Method and Study Area

Fermented dairy samples (yogurt, kefir, paneer) were collected from local retail outlets and small-scale producers in [Insert Location]. A purposive sampling method was used to ensure microbial diversity and relevance to typical consumer sources. Samples were transported under chilled conditions and processed within 24 hours of procurement to preserve native microbial integrity.

Selection and Procurement of Fermented Dairy Products

Five fermented dairy products were selected for this study based on their regional popularity, physicochemical complexity, and susceptibility to microbial spoilage under typical storage conditions. Each product was assigned a reference code to streamline documentation across microbiological assays, physicochemical evaluation, and preservative treatment trials (Table 3).

Samples of each product were procured from three distinct sources: standardized commercial brands available at retail outlets, artisanal vendors operating in local Telangana markets, and household preparations made using traditional methods. The sourcing strategy ensured microbial variability and ecological relevance in the context of preservative application.

Samples were collected aseptically in sterile containers, transported under chilled conditions (4 ± 1°C), and processed within 24 hours of procurement. Basic metadata—including production date, batch ID, and source type—was recorded for each sample, and product codes were consistently referenced throughout data collection and analysis.

Table: Selection of Fermented Dairy Product

Product Code	Fermented Dairy Product	Description
FDP-01	Yogurt (Curd / Perugu)	Semi-solid fermented milk product widely consumed across India.
FDP-02	Kefir	Fermented milk with mixed lactic acid bacteria and yeast; tangy and effervescent.

FDP-03	Paneer	Fresh acid-coagulated dairy solid with high moisture content; spoilage-prone.
FDP-04	Shrikhand	Sweetened and strained yogurt; rich in sugar and vulnerable to yeast/mold.
FDP-05	Misti Doi	Traditional Bengali-style sweet fermented milk; soft-set and moisturerich.

Morphological and Biochemical Identification of Bacteria

Bacterial isolates were first screened based on colony morphology (shape, margin, elevation, color, and texture) and Gram staining. Gram-positive and Gram-negative bacteria were differentiated microscopically and subjected to the following biochemical tests for species-level identification

Table : Purpose of Biochemical Tests

Test	Purpose	Indicative Organisms
Catalase Test	Detects catalase enzyme (bubble formation with H_2O_2)	Staphylococcus spp., E. coli, Bacillus spp.
Oxidase Test	Identifies cytochrome oxidase activity	Pseudomonas spp., Neisseria spp.
Indole Test	Detects tryptophanase activity (red ring with Kovac's)	E. coli, Proteus spp.
Methyl Red Test	Identifies stable acid production from glucose	E. coli, Salmonella spp.
Voges-Proskauer Test	Detects acetoin production	Enterobacter spp., Klebsiella spp.
Citrate Utilization	Determines ability to use citrate as sole carbon source	Klebsiella spp., Citrobacter spp.
Urease Test	Detects urease enzyme (pink color change)	Proteus spp., Yersinia spp.
Triple Sugar Iron (TSI)	Differentiates sugar fermentation and H_2S production	Salmonella spp., Shigella spp., E. coli

All tests were performed using standard protocols as described by Dura (2020) and Aryal (2022). Positive and negative controls were included to validate each assay.

Representative isolates were preserved in 20% glycerol stocks at −20°C for further antimicrobial testing. Fungal isolates were subcultured on PDA slants and stored at 4°C.

IV-RESULTS

Samples and Their Characteristics

A total of five fermented dairy products yogurt (FDP-01), kefir (FDP-02), paneer (FDP-03), shrikhand (FDP-04), and misti doi (FDP-05) were selected and characterized prior to antimicrobial and preservative trials. Products were sampled from three distinct categories: retail brands, artisanal market vendors, and traditional household preparations, ensuring variability in microbial load, compositional integrity, and sensory properties.

To assess their spoilage susceptibility and structural properties, baseline evaluations included pH analysis, texture profiling, and physical observations indicative of microbial risk (e.g., gas formation, sour odor, surface slime).

Physicochemical Observations

Table : Baseline Characteristics of Fermented Dairy Products

Product Code	Product Name	Texture Type	Average pH (n=9)	Sensory Notes (Baseline)	Spoilage Risk
FDP-01	Yogurt	Semi-solid, smooth	4.42 ± 0.05	Slight sourness, surface uniformity	High
FDP-02	Kefir	Grainy, pourable	4.35 ± 0.07	Mild effervescence, slight clump formation	Moderate-High
FDP-03	Paneer	Moist, dense cubes	6.62 ± 0.08	Rancid note in 2/9 samples, pale	High
				discoloration	
FDP-04	Shrikhand	Thick, creamy	4.59 ± 0.06	Sweet odor, glossy surface; yeast-like aroma in 3/9	Moderate
FDP-05	Misti Doi	Soft-set, granulated	4.49 ± 0.09	Slight curdling and gas pockets in artisanal samples	Moderate-High

The data suggest that yogurt and paneer exhibited the most consistent spoilage traits, notably slime formation, acidic shifts, and surface deterioration. Paneer, with its higher baseline pH and moisture

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content, was especially vulnerable to proteolytic bacterial and fungal colonization.

4.1.2 Spoilage Indicators by Product Type

Observations included gas formation in sealed kefir and misti doi containers, surface fungal growth on yogurt and shrikhand, and rancid notes in paneer. These changes aligned with microbial activity under chilled conditions (4 °C) and product handling across sampling sources.

This bar chart distinctly shows FDP-03 (paneer) as an outlier in pH, underscoring its higher spoilage risk from alkaline-tolerant bacteria. This plot highlights paneer's significantly higher pH, reinforcing its vulnerability to bacterial spoilage and underscoring the need for preservative intervention in moisture-rich, non-acidic products (Fig 5).

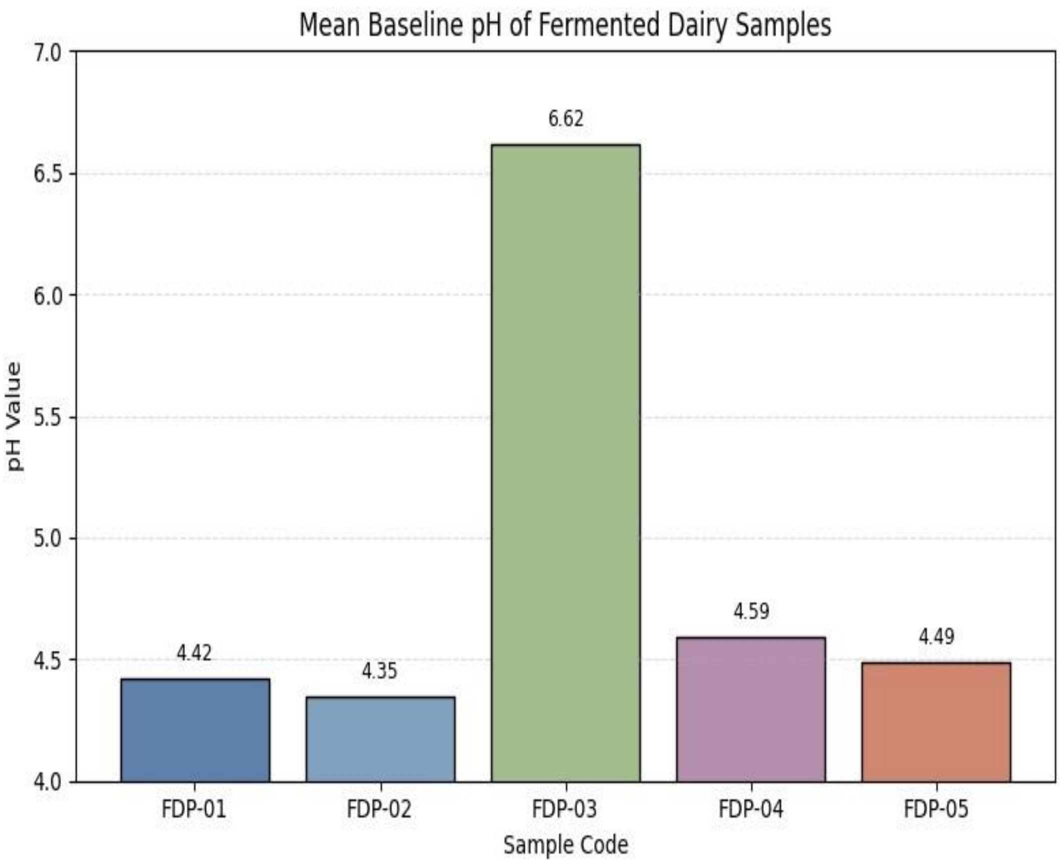

Fig : Mean Baseline pH Values of Fermented Dairy Products

This bar chart distinctly shows FDP-03 (paneer) as an outlier in pH, underscoring its higher spoilage risk from alkaline-tolerant bacteria. This plot highlights paneer's significantly higher pH, reinforcing its vulnerability to bacterial spoilage and underscoring the need for preservative intervention in moisture-rich, non-acidic products.

4.1.3 Preliminary Microbial Load and Surface Observations

Initial microbial screenings on Plate Count Agar indicated TVC (Total Viable Count) ranging from 3.6×10^4 CFU/g in yogurt to 1.2×10^6 CFU/g in paneer, especially from artisanal sources. Yogurt and kefir showed visible fungal patches after 48 hours, while shrikhand and misti doi revealed gas accumulation and curd separation by Day 3.

4.2 Microbial Isolation and Characterization

This section presents the systematic isolation and identification of spoilage microorganisms associated with the five fermented dairy products outlined in Chapter III. The objective was to establish microbial profiles that reflect realistic contamination scenarios under chilled conditions, providing a foundation for evaluating the efficacy of natural preservative treatments.

4.2.1 Microbial Load Assessment

Total Viable Count (TVC) data was generated using standard plate count methodology on nutrient-rich and selective media. Sample dilution (up to 10^{-6}) was followed by surface plating and incubation at 30 ± 2°C for 48 hours. Colony-forming units (CFU/g) were enumerated and transformed to $\log_{10}$ scale for comparative analysis (Fig 6).

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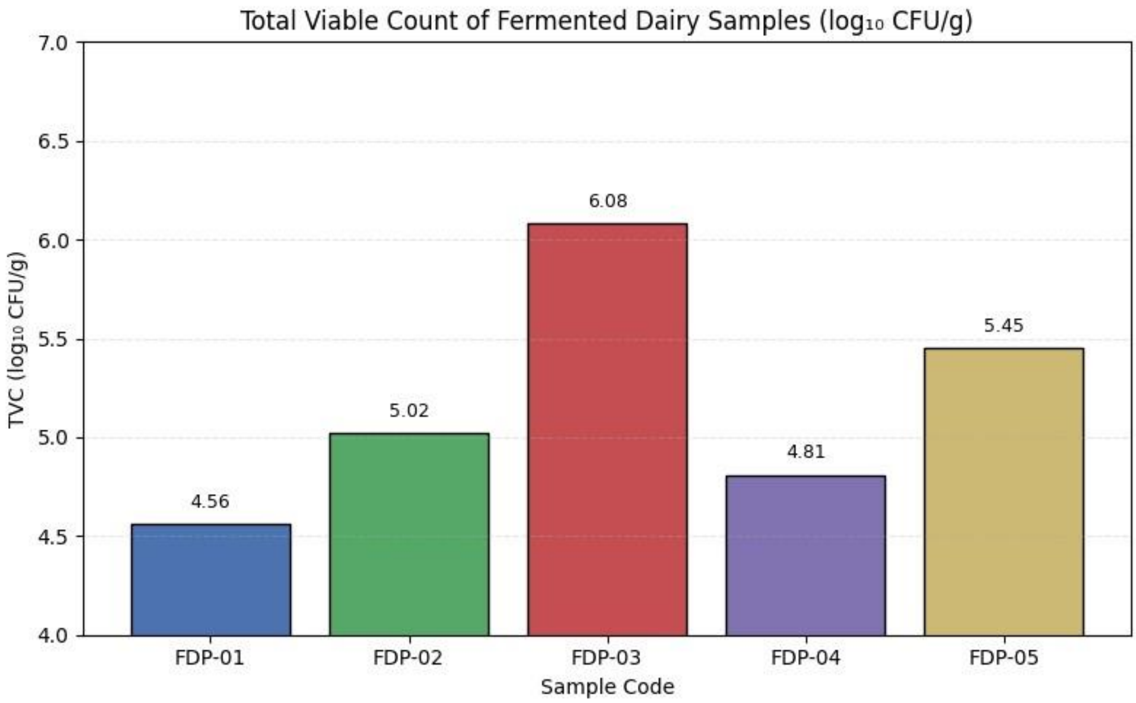

Fig : TVC Profile Across Product Types

Paneer (FDP-03) exhibited the highest microbial load (6.08 log_{10} CFU/g), underscoring its vulnerability due to higher pH and moisture retention. Kefir (FDP-02) and misti doi (FDP-05) also demonstrated elevated counts, consistent with their complex microflora and fermentable substrates. Yogurt (FDP-01) and shrikhand (FDP-04), while mildly acidic, still presented moderate microbial loads, including visible mold development during pretreatment analysis.

4.2.2 Morphological and Biochemical Identification

Recovered isolates were characterized based on colony morphology, Gram staining, and classical biochemical tests. Identification was cross-validated using literature-supported phenotypic markers and selective media outcomes.

Table : Confirmed Spoilage Microorganisms in Fermented Dairy Products

Sample Code	Microorganisms Identified	Type	Key Traits Observed	Gram Reaction
FDP-01	*Candida albicans, Penicillium spp.*	Yeast, Mold	Creamy convex colonies; fuzzy hyphal margins	NA
FDP-02	*Saccharomyces cerevisiae, Pseudomonas fluorescens*	Yeast, Bacterium	Oval yeast cells; green pigmentation colonies	Gram-negative

FDP-03	*Bacillus subtilis, Cladosporium spp.*	Sporeforming bacterium, Mold	Irregular dry colonies; black specks on PDA	Gram-positive
FDP-04	*Staphylococcus aureus, E. coli, Saccharomyces spp.*	Bacteria, Yeast	Mucoid colonies; mild gas formation; creamy dome shapes	Mixed
FDP-05	*Lactic acid bacteria, Coliforms, Yeasts*	Mixed flora	Domed colonies; curd separation in plated samples	Mixed

Note: Identification of *Penicillium spp.* and *Cladosporium spp.* was conducted using cultural features on Potato Dextrose Agar and referenced to the Manoharachary Manual of Fungal Identification (2005) for morphological confirmation.

4.2.3 Biochemical Characterization

Each bacterial isolate underwent classical biochemical assays including catalase, oxidase, indole, citrate utilization, methyl red, and VogesProskauer tests. These allowed differentiation between coliforms, aerobic spoilage organisms (*Pseudomonas spp.*, *Bacillus spp.*), and lactic acid fermenters.

- Catalase activity was positive in all aerobic isolates (*Staphylococcus aureus*, *Bacillus subtilis*)
- Oxidase test helped confirm *Pseudomonas fluorescens* identity
- Indole and MR-VP profiles differentiated coliforms and enteric bacteria
- Urease activity was noted in *Candida albicans*, correlating with substrate alkalization

The dairy products studied presented a diverse range of spoilage organisms, primarily driven by water activity, storage hygiene, and pH buffering capacity. The prominence of yeasts and molds in FDP-01, FDP-04, and FDP05 indicates the need for antifungal components in preservative formulations, while proteolytic and gas-forming bacteria in FDP-02 and FDP-03 warrant antibacterial control.

4.3 Antimicrobial Activity of Natural Preservatives

The antimicrobial efficacy of six natural preservatives garlic extract, ginger extract, clove essential oil, extra virgin olive oil (EVOO), infused olive oil (garlic or thyme), and nisin—was assessed against the spoilage microorganisms isolated from five fermented dairy products. The evaluation was conducted through a series of agar well diffusion assays, followed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations.

CONCLUSION

This research successfully demonstrated that select natural preservatives especially clove essential oil and nisin can significantly improve the microbiological, physicochemical, and sensory stability of five traditionally fermented dairy products during refrigerated storage. Through rigorous comparative analysis across antimicrobial assays, biochemical profiling, sensory evaluation, and spoilage mapping, it was evident that clove essential oil exhibited exceptional antifungal activity, particularly in acidic matrices such as yogurt and shrikhand, while nisin offered targeted Gram-positive bacterial control and structural preservation in products like paneer and misti doi.

Across all matrices, preservative-treated samples achieved extended shelflife thresholds, delayed spoilage onset by 7–10 days, and retained sensory scores above 8.0/10. The inverse correlations observed between microbial load and pH or viscosity further validated the mechanistic role of microbial suppression in quality preservation. Moreover, statistical analyses confirmed the significance and reproducibility of treatment effects across trial sets and product types.

By integrating microbial ecology, product-specific biochemistry, and sensory science, this study provides a scalable preservation strategy rooted in natural compounds. It supports the transition toward sustainable, cleanlabel dairy innovations relevant for both artisanal and commercial applications without compromising consumer preference or safety.

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